PATENT
Attorney Docket No.: 015280-343100US
Client Ref. No.: E-029-98/1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Examiner: Yu, Misook
RYBAK and NEWTON	Art Unit: 1642
Application No.: 09/622,613	DECLARATION OF SUSANNA RYBAK UNDER 37 C.F.R. §1.132
Filed: August 17, 2000	
For: RECOMBINANT ANTI-TUMOR RNASE	

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Susanna M. Rybak, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. I currently hold a position with the National Cancer Institute of the National Institutes of Health as a Senior Investigator in the Developmental Therapeutics Program and I am an inventor on the subject application. My curriculum vitae is attached as Exhibit A. I have a Ph.D. from the University of California San Francisco in endocrinology. In 1986 I was appointed an Assistant Professor of Pathology at Harvard

Medical School where I began work on a series of human serum ribonucleases. While there I demonstrated that a human serum ribonuclease is a potent inhibitor of protein synthesis and since that time I have been working with ribonucleases (RNase) to develop them as therapeutic agents. I have also authored numerous articles on the subject that are listed on my curriculum vitae.

2. I have reviewed the Office Action mailed on November 6, 2002 in the subject application. Our invention relates to new recombinant RNase molecules that can be expressed in bacteria and are cytotoxic. These molecules can be used to selectively kill target cells such as tumor cells. Often, the RNases of the invention are conjugated to a ligand binding moiety to increase cytotoxicity, as described in detail in the subject specification. In this Declaration, I will present additional evidence produced by us that further demonstrates the anti-tumor effect *in vivo* of the RNase alone and when conjugated to a targeting moiety, *e.g,* an antibody.

3. We tested rapLR1 (SEQ ID NO:2) and immunoconjugates comprising rapLR1 on the *in vivo* growth of tumors. Recombinant rapLR1 was expressed and purified from inclusion bodies in BL21(DE3) bacteria that were specifically engineered for expression of toxic proteins. An immunoconjugate containing rapLR1 linked to an anti-mucin antibody was prepared using known techniques (*e.g.*, Newton and Rybak, In: *Methods in Molecular Medicine*, Vol 25: *Drug Targeting: Strategies, Principles and Applications*, p. 27-35, 2000, Francis & Delgado, Eds.). Briefly, rapLR1 was treated with a 2.9-fold molar excess of SPDP for 30 minutes to obtain a substitution of 0.9 to 1.2 mol SPDP/mol rapLR1. After excess SPDP was removed by PD10 chromatography, the rapLR1-2-pyridyl disulfide derivative was treated with 2 mM DTT for 1 h to reduce the 2-pyridyl disulfide bond. The anti-mucin antibody was incubated with a 55 molar excess of 2-IT and final concentration of 2 mM DTNB in 100 mM sodium borate, pH 8.5 for 1 h. Immediately after the removal of excess 2-IT and DTNB from the antibody solution, and DTT from the rapLR1 solution by G25 chromatography, the two modified proteins

(16:1 rapLR1:antibody molar ratio) were combined and incubated overnight. The conjugate was separated from unconjugated rapLR1 by chromatography on a TSK 3000 SW column.

4. In one experiment, rapLR1 and an immunoconjugate of rapLR1 and the anti-MUC1 antibody (HMFG1) were tested for the ability to inhibit metastasis in a murine breast adenocarcinoma model. About 10^5 DA-3 breast adenocarcinoma cells were injected intravenously on day 0 into Balb C mice. Intravenous treatments with rapLR1 or the rapLR1 immunoconjugate were initiated on Day 3 following injection. Treatment continued for 5 consecutive days. The treatment protocol was as follows: once per day, HMFG1-rapLR1 conjguate was administered at 50 μg/dose, for a total treatment of 12.5 mg/kg; rapLR1 was administered in an amount equivalent to that in the conjugate, *i.e.*, 12 μg/dosc for a total treatment of 3 mg/kg. Control animals were injected with PBS or HMFG1 alone. At day 31, animals were euthanized, the lungs removed and fixed in Bouins' fixative, and the lung metastases were counted. The results (Exhibit B) showed that the numbers of metastases were reduced in those animals that received rapLR1. Further, the animals that received the immunoconjugate had even fewer metastases. Thus, both rapLR1 alone and as a component of an immunoconjugate reduced metastasis.

5. The effects of rapLR1 and HMFG1-rapLR1 were also tested on the growth of 4TI-MUC1 tumors that were implanted into the mammary fat pad of SCID mice. About 10^5 4TI breast adenocarcinoma cells were injected into the mammary fat pad of SCID mice. RapLR1 and HMFG1-rapLR1 conjugates were administered intravenously beginning on day 3. Treatment was continued 3 times per week for 2 weeks. HMFG1-rapLR1 conjugate was administered at 100 μg /dose, 3 times per week for 2 weeks, total treatment was 30 mg/kg; rapLR1 was administered at an amount equivalent to that in the conjugate, 24 μg/dose, total treatment was 7.2 mg/kg). Tumor volumes were measured 3 times/week. The results (Exhibit C) show that rapLR1 by itself and when linked to an anti-MUC1 antibody inhibited growth of the tumor cells.

6. RapLR1-anti-CD22 have has also been tested *in vivo* in an aggressive model of disseminated murine lymphoma and shown to have anti-tumor activity. These results have recently been published (Hursey *et al.*, *Leukemia and Lymphoma* 43:953-959, 2002); a copy of the publication is provide in Exhibit D. In this model, Daudi lymphoma cells (5 x 10^6) were injected either intraperitoneally (ip) or intravenously (iv). The animals that received ip injections of tumor cells were then treated for 5 consecutive days beginning on day 1 with ip injections of either PBS, 80 μg LL2 plus 20 μg rapLR1, or 100 μg of LL2-rapLR1 conjugate. The conjugate was also effective in treating mice with a more advanced tumor burden. Those animals that received iv injections of tumor cells were treated for 5 consecutive days beginning at six days after tumor cell injection. The animals were treated with either PBS, 80 μg LL2 plus 20 μg rapLR1, or 100 μg of LL2-rapLR1 conjugate. The results (Table III, page 957) showed that those animals that received the LL2-rapLR1 conjugate exhibited enhanced survival. The conjugate was also effective in treating mice with a more advanced tumor burden (i.v. injection with treatment begun six days after tumor cell injection).

7. In summary, the data presented in this Declaration further demonstrate that rapLR1 by itself or as a component of an immunoconjugate is effective *in vivo* as an anti-tumor agent in multiple tumor models.

8. All statements herein made of my own knowledge are true and statements made on information or belief are believed to be true.

Dated: April 7, 2003

Susanna M. Rybak, Ph.D.

Attachments: Exhibits A-D